Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem

Sean P. Gordon, Vijay S. Chickarmane, Carolyn Ohno[1], and Elliot M. Meyerowitz[2]

Division of Biology 156-29, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125

Contributed by Elliot M. Meyerowitz, July 27, 2009 (sent for review June 3, 2009)

A central unanswered question in stem cell biology, both in plants and in animals, is how the spatial organization of stem cell niches are maintained as cells move through them. We address this question for the shoot apical meristem (SAM) which harbors pluripotent stem cells responsible for growth of above-ground tissues in flowering plants. We find that localized perception of the plant hormone cytokinin establishes a spatial domain in which cell fate is respecified through induction of the master regulator *WUSCHEL* as cells are displaced during growth. Cytokinin-induced *WUSCHEL* expression occurs through both CLAVATA-dependent and CLAVATA-independent pathways. Computational analysis shows that feedback between cytokinin response and genetic regulators predicts their relative patterning, which we confirm experimentally. Our results also may explain how increasing cytokinin concentration leads to the first steps in reestablishing the shoot stem cell niche in vitro.

clavata | wuschel | computational modeling

Plants ranging from the small weed *Arabidopsis* to the giant sequoia tree, maintain growth of stems, leaves, flowers, and branches through the action of stem cells. In the model plant *Arabidopsis*, as in other flowering plants, stem cells which give rise to above-ground tissues reside in a structure termed the shoot apical meristem (SAM) (1, 2). The *Arabidopsis* SAM is composed of three functionally distinct zones. The central zone (CZ) at the tip of the SAM harbors pluripotent stem cells which are necessary for the indeterminate growth and development of the plant. As the plant grows, CZ cells become either multipotent peripheral zone (PZ) cells on the sides of the meristem, capable of differentiating to leaf and flower primordia, or multipotent rib meristem (RM) cells beneath, which can differentiate to the cell types of the stem (3). Positions of zones within the meristem are maintained even as individual cells are displaced from the CZ through the PZ and RM into differentiating tissues. Molecular mechanisms by which meristematic zones are maintained as cells comprising these domains change remains a fundamental question in plant biology (1, 4). One mechanism involves the transmembrane receptor kinase CLAVATA1 (CLV1), expressed in cells of the RM (5). Its ligand, the extracellular peptide product of the *CLAVATA3* (*CLV3*) gene, is produced in the CZ (6), and when it signals the RM cells, they reduce the activity of the *WUSCHEL* (*WUS*) gene, which codes for a homeodomain transcription factor also expressed in the RM (7, 8). WUS activity is nonautonomously necessary for the maintenance of the CZ cells as pluripotent stem cells, and therefore for persistence of the SAM (9). Loss of CLV3 activity causes enlargement of the CZ by conversion of PZ cells on the PZ-CZ border to CZ cells within hours, followed by enlargement of the SAM through increased cell division or reduced differentiation, or both, over days (10).

Multiple lines of evidence show that the plant hormone cytokinin is involved in the *CLV/WUS* circuit, as well as SAM formation, maintenance and growth (1). Cytokinins stimulate the formation of new shoot apical meristems in culture (11). Cytokinin application rescues the *SHOOTMERISTEMLESS (STM*) mutant, which lacks the ability to maintain the SAM (12), and *STM* induces cytokinin biosynthetic genes (13, 14). Mutants for the *LOG* gene of rice, which encodes an enzyme that catalyzes the production of active cytokinins in the apical stem cell region of the SAM, have reduced shoot meristem size and prematurely terminate floral meristems (15, 16). Cytokinins act via receptors of the histidine kinase class (AHK2, 3, and 4), which when activated transfer phosphoryl groups to histidine phosphotransfer proteins (HPTs) and thence to two classes of *Arabidopsis* response regulators (ARRs) (17, 18). The Type-B ARRs activate transcription of cytokinin-induced target genes; Type-A ARRs negatively regulate cytokinin signaling (18–20). WUS has recently been shown to repress the genes for Type-A ARRs, thus likely increasing cytokinin signaling (21). Furthermore, overexpression of a Type-A ARR reduces *WUS* RNA levels, and can mimic the *wus* mutant phenotype (21) [SAM termination (22)]. Cytokinin treatment induces *CLV* loss-of-function phenotypes and causes increased *WUS* and decreased *CLV1* expression (23, 24).

In this study, we reveal multiple feedback loops between cytokinin response and WUS which influences gene expression and patterning within the *Arabidopsis* SAM. We use live imaging and an array of reporters to show that cytokinin perception and response is localized within the SAM where it regulates the pattern of *WUS*, a key positive genetic regulator of stem cell fate. We demonstrate that cytokinin signaling activates *WUS* expression through both *CLV*-dependent and *CLV*-independent pathways. We develop a computational model of cytokinin signaling which shows that feedback between cytokinin response and key genetic regulators determines the probability that a cell will express *WUS*. Given that *WUS*-expressing cells promote stem cell number and recent evidence that stem cells are a source of active cytokinins (16), our results may support a positive feedback loop between stem cells and underlying RM cells that maintains the organization of the SAM as stem cells are displaced during growth.

Results

CLV-Dependent and CLV-Independent Regulation of WUS by Cytokinin Signaling. Prior studies have shown that *WUS* expression within the SAM is partly restricted spatially through negative feedback from the *CLV* pathway shown in Fig. 1*A*. Recent studies have shown that treatment of plants with high levels of cytokinin leads

Author contributions: S.P.G. and V.S.C. designed research; S.P.G. performed research; C.O. contributed new reagents/analytic tools; V.S.C. performed the computational analysis and model building; S.P.G., V.S.C., and E.M.M. analyzed data; and S.P.G., V.C., and E.M.M. wrote the paper.

The authors declare no conflict of interest.

See Commentary on page 16016.

[1]Present address: Developmental Biology Unit, EMBL, Meyerhofstrasse 1, 69117 Heidelberg, Germany.

[2]To whom correspondence should be addressed. E-mail: meyerow@its.caltech.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0908122106/DCSupplemental.

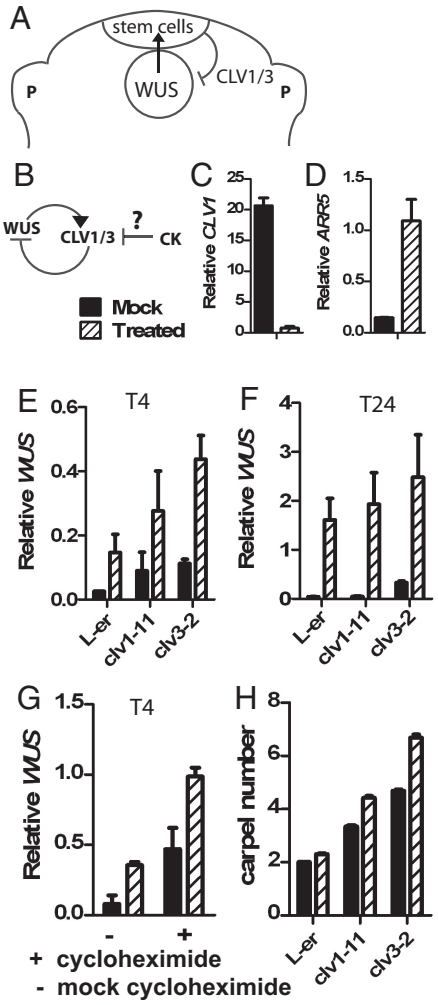

Fig. 1. *CLV*-independent regulation of *WUS* by cytokinin. (*A*) *WUS*/*CLV* interactions in a cross section of the SAM. *WUS* expression in RM cells promotes stem cell fate in overlying cells. Stem cells in turn secrete diffusible CLV3 ligand that binds to its receptor CLV1 in the RM leading to *WUS* downregulation. P labels organ primordia. (*B*) Hypothetical circuit in which cytokinin (CK) treatment leads to higher levels of *WUS* through suppression of *CLV1*. (*C* and *D*) *CLV1* (*C*), or *ARR5* (*D*) transcript after 24 h of mock or cytokinin treatment. (*E* and *F*) relative *WUS* transcript in wild-type, *clv1–11*, and *clv3–2* seedlings after mock or cytokinin treatment for (*E*) 4 h, or (*F*) 24 h ($P < 0.05$). (*G*) Cytokinin induction of *WUS* for 4 h in absence (−) or presence (+) of 30 min cycloheximide (10 μM) pretreatment. (*H*) Enhancement of carpel number in cytokinin treated *clv1* and *clv3–2* mutants compared to wild type (two-way ANOVA, F = 81, $P < 0.0001$). qRT-PCR error bars indicate SEM from three biological replicates.

to *CLV* loss-of-function phenotypes and causes increased *WUS* and decreased *CLV1* expression (23, 24). These data lead to a qualitative model in which cytokinin treatment increases *WUS* expression through suppression of *CLV*-mediated negative feedback on *WUS* levels (Fig. 1*B*). To test this starting hypothesis, we quantified the effect of cytokinin treatment on *CLV1* and *ARR5* transcription, as measured by quantitative reverse transcriptase PCR (qRT-PCR). As previously reported (20, 23), 24 h of cytokinin treatment reduced *CLV1* RNA levels and increased RNA for the Type-A ARR, *ARR5* (Fig. 1 *C* and *D*). To test whether repression of the *CLV* pathway is the only mechanism of *WUS* induction by cytokinin (23), we performed cytokinin treatments in a *clv1–11* loss-of-function mutant. At 4 and 24 h after cytokinin treatment, *WUS* RNA increased in both wild-type and mutant lines compared to mock-treated samples (Fig. 1*E* and *F*); by 24 h *WUS* transcript was increased approximately 40-fold in both genotypes. Pretreatment of the plants with the protein synthesis inhibitor cycloheximide did not prevent this induction (Fig. 1*G*), suggesting a direct effect. Cytokinin treatment also induced *WUS* transcript accumulation in a *clv3–2* loss-of-function mutant background, suggesting that induction in the *clv1–11* mutant is not due to redundant function of related CLV3-dependent kinases active in the SAM, such as BAM1, 2 and 3 (25), CLV2 (26), or CORYNE (27). We observed greater phenotypic enhancement of floral organ number (an indicator of increased floral meristem size and stem cell activity) by cytokinin in *clv1* and *clv3* mutants compared with wild type, suggesting a synergistic interaction between cytokinin and *CLV* loss of function (Fig. 1*H*, two-way ANOVA, F = 81, $P < 0.0001$). In contrast to wild type, cytokinin (benzylaminopurine, BAP) treatment of *clv* mutants resulted in massive enlargement of the SAM and floral meristems (Fig. S1). Similar fold induction of *WUS* transcript in wild type and *clv* mutants after continuous cytokinin treatment reveals the existence of CLV-independent mechanisms of cytokinin-induced *WUS* expression (Fig. 1 *E–G*). However, greater phenotypic enhancement in *CLV* loss-of-function background indicates that the *CLV* pathway limits the effect of transient perturbations in cytokinin signaling and therefore indicates that there are also *CLV*-dependent effects (Fig. 1*H*; see Computational Modeling in *SI Appendix*).

Feedback Between Cytokinin Signaling and the *WUS*/*CLV* Circuit Influences Patterning of Gene Expression. After 24 h, *CLV1* and *ARR5* transcript levels were altered at low cytokinin concentrations. However, increase in *WUS* transcript occurred only at high concentration (Fig. 2*A*). Finer dilutions showed a steep rise in *WUS* transcript and corresponding decrease in *ARR5* beginning at 400 μM and peaking near 600 μM (Fig. S1). We reasoned that the observed increase of *WUS* transcript after cytokinin perturbation could be indicative of a role for endogenous cytokinin response in influencing the pattern of *WUS* expression. Cytokinin response can be visualized at high resolution using a synthetic reporter, *pTCS*::*GFP* (28), which reports downstream activation of the cytokinin signaling pathway. Our data showing the sharper and higher threshold of cytokinin required for *WUS* induction as compared to *ARR5* (Fig. 2*A*) indicates that *WUS* should closely overlap spatially with high levels of cytokinin response and drop off sharply in cells less responsive to cytokinin within the SAM. Consistent with this hypothesis, the *pTCS*::*GFP* reporter for cytokinin response was activated in a similar domain to *WUS* (Fig. 2 *B–D*). *pTCS*::*GFP* expression mirrored temporal dynamics of *WUS* reporter expression during floral meristem development and SAM regeneration in culture (Fig. S2), consistent with a model where *WUS* is spatially regulated by cytokinin signaling during development (29).

Previous studies have shown that WUS directly suppresses the transcription of a subset of Type-A ARRs involved in negative feedback on the cytokinin signaling pathway. Thus WUS likely increases cytokinin signaling (21). From the perspective of a gene regulatory network, regulation of *WUS* levels by cytokinin signaling, either by *CLV*-dependent or independent pathways, completes a positive feedback loop between cytokinin signaling and *WUS*. Cytokinin-induced increase in *WUS* levels leads to greater suppression of Type-A ARRs which leads higher cytokinin signaling and thus higher *WUS* levels. To better understand the nature of these potential positive feedback loops we used computational modeling (see Computational Modeling in *SI Appendix* for details) to plot predicted steady state values of *WUS* as a function of cytokinin signaling (Fig. 2 *F–J*). We first considered the three hypothetical networks shown in Fig. 2*E*. In the circuit displayed in Fig. 2*E* (1), cytokinin signaling regulates *WUS* through suppression of *CLV1* alone. Alternatively, in the

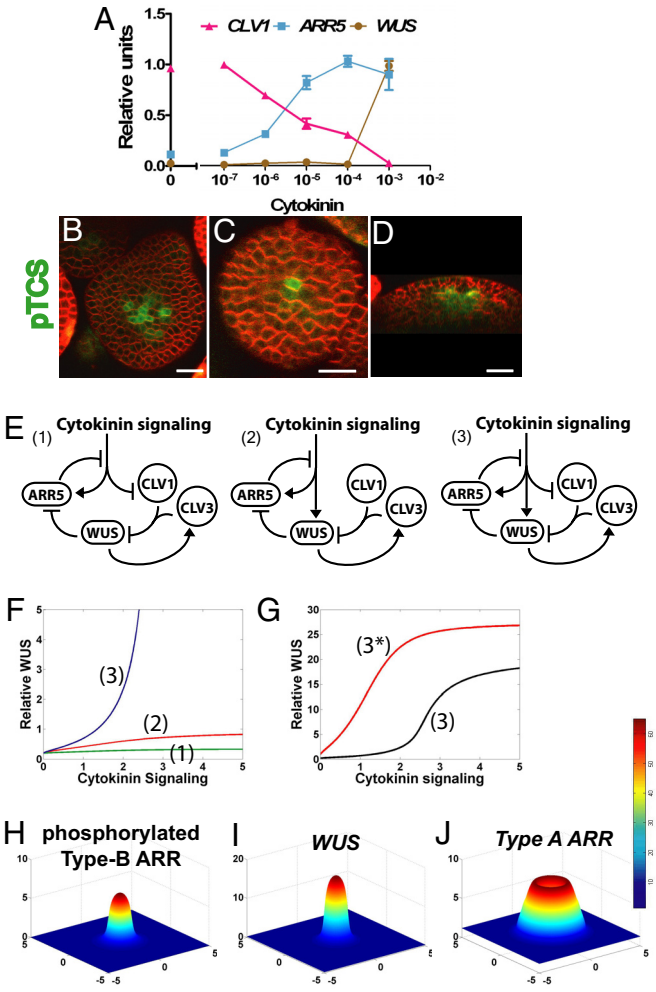

Fig. 2. Feedback between cytokinin signaling and the *WUS/CLV* circuit influences patterning of gene expression. (*A*) Relative *CLV1*, *ARR5*, and *WUS* RNA transcript at varying cytokinin concentrations. (*B–D*) *pTCS*::*GFP* expression in the SAM (*B*), early flower bud (*C*), or cross section of SAM (*D*). (*E*) (1) Cytokinin activates *WUS* through suppression of CLV1 or (2) a CLV-independent pathway or (3) through both mechanisms. (*F*) Predicted steady state *WUS* levels at varying levels of cytokinin signaling for circuits (1, green line), (2, red line), and (3, blue line). (*G*) Steady state *WUS* levels for network (3) including CLV negative feedback compared to network 3 lacking the CLV pathway (3*). (*H–J*) spatial distribution of phosphorylated B-type ARR (*H*), *WUS* (*I*), or *Type-A ARR* (*J*) for network 2E (3). Axis of the plots correspond to a section of the meristem using arbitrary units in which 0,0 marks the center of the meristem. Error bars indicate SEM from two biological replicates. (Scale bars, 20 mm.)

network displayed in Fig. 2*E* (2) *WUS* transcription is activated through a *CLV*-independent mechanism. In contrast, Fig. 2*E* (3) shows a network in which *WUS* is regulated by both *CLV*-dependent and independent mechanisms, as suggested from our experiments. Plots in Fig. 2*F* show that increases in *WUS* in models considering *CLV*-dependent or *CLV*-independent regulation of *WUS* alone are limited. In contrast, when cytokinin signaling activates *WUS* through both mechanisms a massive increase in *WUS* occurs. In network Fig. 2*E* (1), *WUS* increase is bounded as the maximum level of *WUS* which can be achieved is equivalent to the *clv1* mutant, a roughly 3–4-fold increase in *WUS* compared to wild type as shown in Fig. 1E (mock treated L-er versus mock treated clv1–11). In network (2) *WUS* increase is bounded by the presence of negative feedback from the *CLV* pathway. Higher *WUS* levels leads to higher levels of CLV3 that suppresses *WUS* transcription. In comparison, in network (3) *WUS* can be induced an order of magnitude greater than in the first two cases, similar to the experimentally observed an approximate 40-fold increase. Suppression of *CLV1* transcription by cytokinin signaling allows *CLV*-independent induction of *WUS* to occur with less suppression from the *CLV* pathway. The effect of functional CLV negative feedback is shown in Fig. 2*G*. Plots in Fig. 2*G* show that in the absence of CLV negative feedback (3*), WUS is induced at lower levels of signaling than in circuits in which the CLV pathway is present (3). Negative feedback on cytokinin signaling through Type-A ARRs also contributes to the high threshold required for WUS induction.

We used our computational model (see Computational Modeling in *SI Appendix*) to predict the pattern of components within the circuit displayed in Fig. 2*E* (3), given our data showing a central peak of cytokinin signaling within the SAM (Fig. 2 *B–D*). Plots of predicted steady state values of activated B-Type ARR, *WUS*, and the Type-A ARR, *ARR5* (known to be suppressed by WUS) are show in Fig. 2 *H–J*. These plots show that *WUS* is predicted to closely overlap with cytokinin signaling as we observe experimentally. In contrast, *ARR5* is predicted to be suppressed where cytokinin signaling is highest and expressed strongly in a peripheral ring-shaped domain.

Distribution of Cytokinin Receptor, Cytokinin Response, and *WUS* Correlate in Individual Cells Where *ARR5* Is Suppressed. To test the predictions of our model, we experimentally determined the relative spatial expression of *WUS* and the Type-A ARR, *ARR5*. We observed that a transcriptional reporter for *ARR5* was suppressed in the *WUS* domain but expressed strongly in adjacent cells forming a ring-like expression pattern (Fig. 3 *A–C*), consistent with the predictions of our computational model. Activation of *WUS* expression by cytokinin perturbation and overlap of WUS expression with the reporter of downstream cytokinin response, *pTCS*::*GFP*, suggested that endogenous cytokinin response might act as a positional cue for patterning *WUS* transcription.

Upstream of WUS function, cytokinin response is governed by cytokinin receptor availability and the local concentration of cytokinin. Therefore, localized cytokinin response within the center of the SAM could be indicative of either a higher local concentration of cytokinin or increased perception of cytokinin in these cells through localized receptor expression. To investigate the latter possibility we determined the distribution of cytokinin receptor expression within the SAM. Indeed, fluorescent reporters for the cytokinin receptor *AHK4* (30), and *WUS* transcription were expressed in overlapping domains within the SAM and were correlated in individual cells (Fig. 3 *D–H*). *AHK4* and *WUS* reporters were similarly regulated during floral meristem development, expanded similarly in the *clv3–2* mutant and were both altered in super-enlarged cytokinin-treated *clv3–2* SAMs (Fig. 3 *I–L* and Fig. S1). *AHK4* and *WUS* reporters also overlapped during SAM regeneration in culture. *AHK4* reporter was induced in cultured cells during pretreatment on auxin-rich medium known to promote regeneration (Fig. S2). Transfer to cytokinin-rich medium results in *WUS* induction (29) in cells marked by the *AHK4* reporter in developing SAMs (Fig. S2).

Cytokinin Regulates Domain of Cytokinin Signaling Output, *WUS* and *CLV3* Expression. The above results suggested that cytokinin receptor distribution initiates a gradient of cytokinin signaling peaking within the center of the SAM and which patterns *WUS* expression in multiple contexts. This model predicts that treatment of plants with exogenous cytokinin would extend sufficient signaling to cells farther from the center of the SAM which have lower levels of receptor, causing *WUS* activation in an expanded domain. Indeed, live imaging before and after 12 h of cytokinin treatment showed expansion of the *WUS* reporter expression domain (Fig. 4 *A–D* and Fig. S3). We observed respecification of

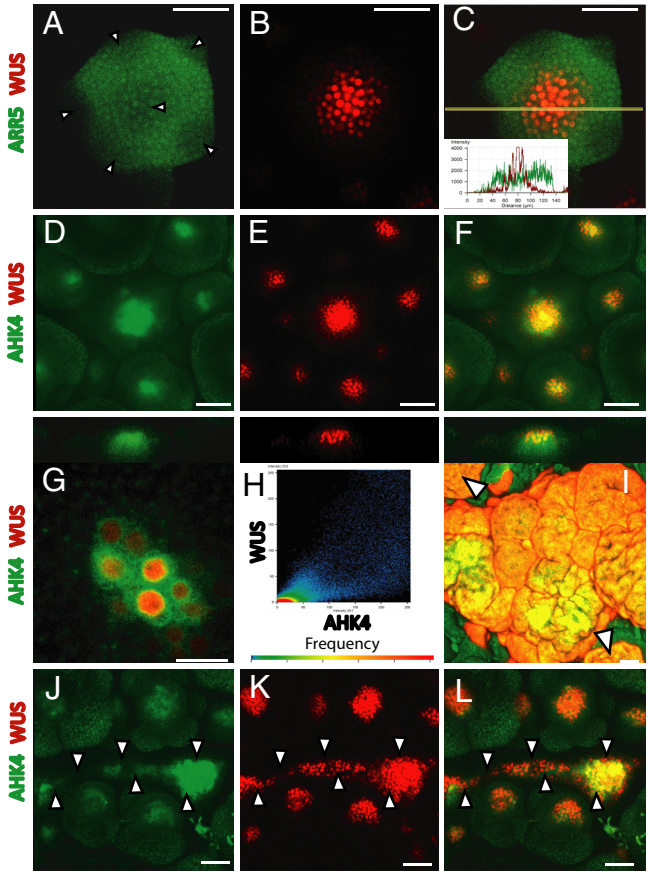

Fig. 3. *AHK4* and *WUS* expression correlate in individual cells where *ARR5* is suppressed. (*A–C*) *ARR5* (green) reporter down regulation within the *WUS* domain (red) and organ primordia (*AHP6* domain, Fig. S3). Inset in (*C*) plots *ARR5* and *WUS* intensity (yellow line indicates profile). (*D–F*) Cytokinin receptor (*AHK4*, green) and *WUS* reporter (red) overlap within the SAM (center) or floral meristems (peripheral). Cross sections displayed below. (*G*) *AHK4* and *WUS* overlap in single cells. (*H*) pixel intensity of *AHK4* (*x* axis) and *WUS* (*y* axis) reporters in wild-type flowers (correlation coefficient R = 0.79, upward trend indicates positive correlation). (*I*) *WUS* (red) and *AHK4* (green) in cytokinin treated *clv3–2* SAM and floral meristems (arrows) compared to untreated *clv3–2* mutants (*J–L*). [Scale bars, 20 μm except for 10 μm in (*G*) and 100 μm in (*I*).]

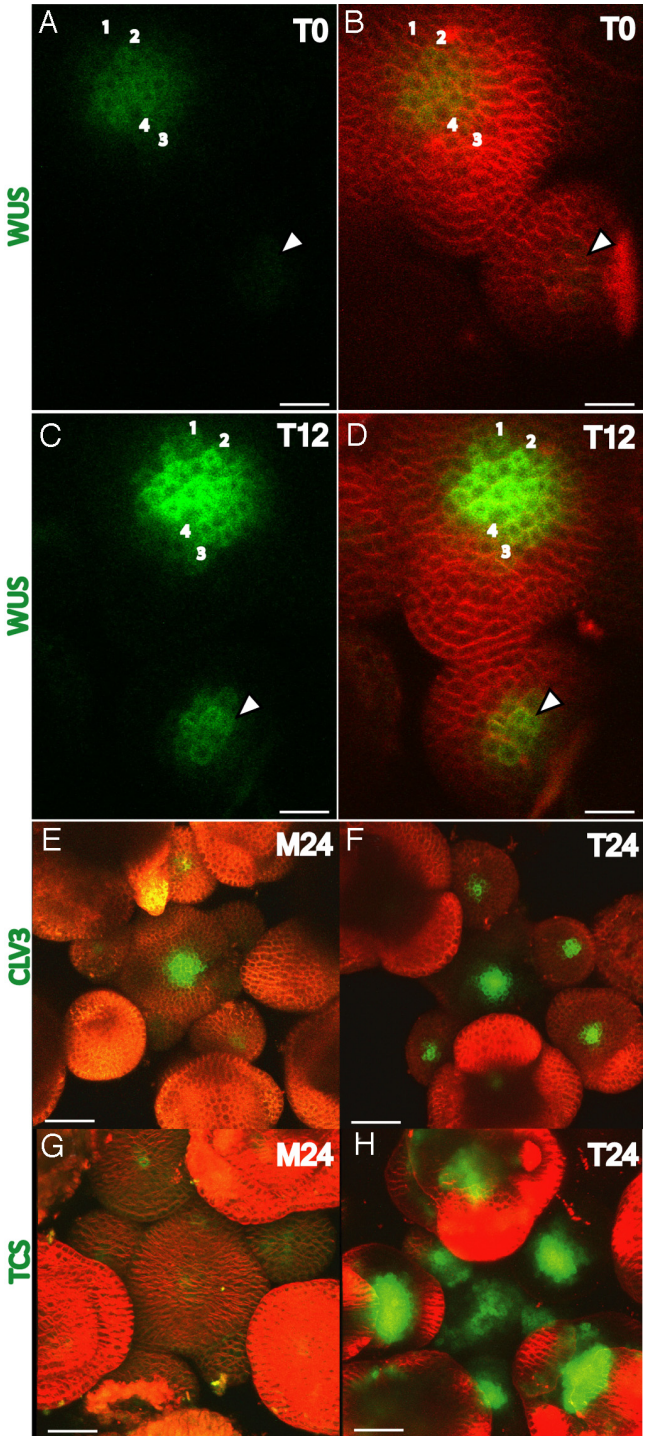

Fig. 4. Cytokinin regulates domain of cytokinin signaling output, *WUS* and *CLV3* expression. (*A–D*) live imaging of *WUS* reporter (green) before (*A* and *B*) and after 12 h of cytokinin treatment (1mM BAP) (*C* and *D*). Numbering in (*A–D*) registers cells in (*A* and *B*) to the same cells in (*C* and *D*) after 12 h. Arrow marks floral meristem. (*E*) *CLV3* reporter (green) in plants after 24 h of mock treatment ($n = 5$) or (*F*) cytokinin treatment ($n = 5$). (*G*) *pTCS*::*GFP* reporter (green) after 24 h of mock treatment ($n = 5$) as compared to (*H*) 24 h of cytokinin treatment ($n = 5$). Membranes are marked with FM4–64 dye (*A–D* and *G* and *H*) or 29–1 membrane YFP marker (*10*) (*E* and *F*). (Scale bars, 50 μm.)

cells that previously did not express *WUS*, indicating that *WUS* domain expansion was not solely due to increased cell division in the RM (Fig. 4 *A–D*). Therefore, similarly to recruitment of surrounding cells into CZ cells after loss of CLV3 activity (*10*), cytokinin increase leads to recruitment of surrounding cells into WUS expressing cells. After 24 h of cytokinin treatment we observed expanded *pTCS*::*GFP* and *pCLV3*::*GFP-ER* reporter expression within inflorescence and floral meristems which was not observed in mock treated samples (Fig. 4 *E–H*). Cytokinin treatment was also sufficient to induce ectopic *WUS* expression, but only in cells which express high levels of cytokinin receptor (Fig. S3) (30).

Cytokinin-Induced Increase of *WUS* Transcript and Related Meristem Phenotypes Requires a *CLV*-Independent Pathway Through an *AHK2*/*AHK4*-Dependent Mechanism. Our results suggest that cytokinin receptor distribution controls the distribution of cytokinin response and thereby influences the pattern of *WUS* expression within the SAM. To determine whether the induction of *WUS* by cytokinin perturbation requires functional cytokinin receptors we quantified *WUS* levels in cytokinin receptor loss-of-function backgrounds (Fig. 5*A*). Of the three characterized cytokinin receptors only *AHK2* and *AHK4* mutants showed significantly lower relative *WUS* transcript levels after cytokinin treatment as compared to wild type (one-way Anova, $F = 42$, $P < 0.05$, Fig.

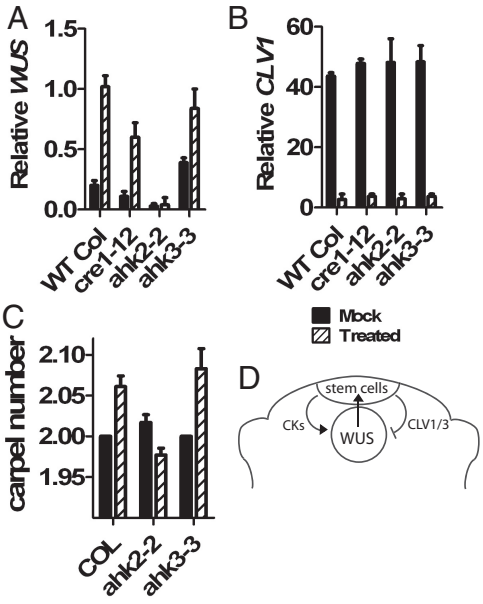

Fig. 5. Cytokinin regulates *WUS* expression through an *AHK2*/*AHK4* dependent mechanism while *CLV1* suppression has no requirement for individual receptors. (*A*) Relative *WUS* or (*B*) *CLV1* transcript levels in wild-type and individual cytokinin receptor mutants after 24 h of mock treatment or cytokinin treatment. (*C*) Cytokinin-induced *clv* mutant-like carpel number phenotypes in wild type (COL), *ahk2–2*, and *ahk3–3* mutants. (*D*) Hypothetical positive feedback between apical stem cells and RM cells. Apical stem cells produce active cytokinins (CKs) perceived by RM cells expressing sufficient cytokinin receptor to activate *WUS* expression. *WUS*, in turn, promotes stem cell fate in apical cells. Negative feedback from the *CLV* pathway is also shown.

5*A*). In contrast, relative *WUS* transcript was not significantly different between cytokinin treated *AHK3* mutant and wild-type samples. Consistent with this observation, *clv* mutant-like phenotypes associated with *WUS* misregulation after cytokinin treatment were not observed in the *ahk2–2* mutant but were observed in the *ahk3–3* mutant similar to wild-type plants (Fig. 5*C*). In comparison to *WUS*, cytokinin-induced suppression of *CLV1* transcript occurred at a similar magnitude in all backgrounds (Fig. 5*B*). These data indicate that the AHK2 and AHK4 receptors are required for cytokinin-induced up-regulation of *WUS* transcript levels and associated *clv* mutant-like phenotypes. In contrast, cytokinin-induced suppression of *CLV1* transcript does not have specific requirements for individual receptors. The fact that *CLV1* was suppressed in all backgrounds but *WUS* up-regulation and cytokinin-induced *clv* mutant-like phenotypes were blocked in the *AHK2* mutant (*ahk2–2*), suggests that induction of *clv* mutant-like phenotypes by cytokinin treatment requires a *CLV*-independent pathway of *WUS* induction by cytokinin.

Discussion

We propose that within the shoot meristem a standing gradient of cytokinin response, dictated in part by cytokinin receptor distribution, acts as spatial reference to inform cells of their position. As cells move into the RM, high cytokinin signaling triggers cell respecification through induction of *WUS*. Given recent evidence for localized production of active cytokinins in shoot stem cells (15), our results support a feedback principle for maintenance of stem cell niche organization during growth (Fig. 5*D*). Stem cells specify RM cell fate through production of active cytokinins which are locally perceived by underlying cells leading to induction of the master regulator *WUS*. In turn, *WUS*-expressing cells in the RM promote stem cell fate in overlying cells. Such positive reinforcement between these two domains could maintain their juxtaposition as the apical stem cells are displaced during post embryonic growth.

During in vitro reestablishment of the shoot stem cell niche in tissue culture, high cytokinin signaling triggers induction of ectopic *WUS* expression leading to stem cell fate in surrounding cells (29). Ectopic *WUS* expression is sufficient for induction of shoot tissues and *WUS* is functionally required for de novo formation of the SAM in vitro (29, 31, 32). Therefore induction of *WUS* through cytokinin treatment may be a key link in triggering the formation of shoot tissues in culture. Cytokinin is sufficient to induce *WUS* expression in the stele of root explants where *AHK4* is expressed (Fig. S3). Auxin pretreatment of tissue explants is used to enhance the efficiency of regeneration in culture (29). Our results show that auxin treatment leads to callus formation associated with broad up-regulation of the *AHK4* receptor (Fig. S2). Thus, it is possible that the ability of auxin pretreatment to enhance regeneration in culture is mediated through the up-regulation of cytokinin receptor expression. This enables a larger population of cells to be competent to respond to cytokinin and trigger high cytokinin signaling required for up-regulation of *WUS* when explants are subsequently induced with cytokinin. The ability of cytokinin signaling to alter cell fate through induction of *WUS* expression is a common thread that links in vitro regeneration of shoot tissues and normal shoot development.

Cytokinin receptors appear to be redundant in many contexts (18). One known example of cytokinin receptor specificity is in the control of leaf senescence in *Arabidopsis* that specifically requires the AHK3 receptor (33). We demonstrate that cytokinin-induced up-regulation of *WUS* transcript is mediated primarily through AHK2 and AHK4 dependent pathways and does not require the AHK3 receptor. Furthermore, cytokinin-induced *clv* mutant-like phenotypes associated with *WUS* misregulation are not observed in the *ahk2–2* mutant. Published microarray data suggests that expression of the cytokinin receptors is not involved in a positive feedback loop with *WUS*, as transient overexpression of *WUS* leads to reduction of *AHK4* levels and does not significantly alter *AHK2* levels (21).

Previous computational models have addressed how *WUS* expression is confined to a small number of cells within the SAM (34), and recently how the *CLV* and *WUS* cell populations maintain each other through feedback between WUS and the CLV pathway (35). In the first study, several alternative hypotheses to maintain *WUS* spatial pattern were considered. A model that assumed a localized activation of *WUS* was best able to reproduce its experimentally observed pattern. In this study we propose that cytokinin is a potential candidate for an activator similar to the hypothetical activator described in the above study, which is locally perceived within the SAM and thus influences the *WUS* expression pattern. Our study represents an attempt to computationally model the cytokinin signaling pathway. We then integrate this model with components of the *WUS*/*CLV* feedback system. Hence, our model is similar to previous studies (34, 35) in how components of the *WUS*/*CLV* pathway interact to spatially maintain their regions within the SAM. However, unlike previous studies, the model reported here provides an understanding of hormonal feedback on gene expression, through a detailed study of the cytokinin perception network. This study (see Computational Modeling in *SI Appendix*) reveals the functionality of several nested feedback loops which suggest a threshold-dependent activation of *WUS*, as a function of cytokinin. The threshold for activation occurs because sufficient cytokinin must build up before: (*i*) negative feedback of the CLV pathway is suppressed; (*ii*) sufficient WUS is accumulated to repress Type-A ARRs and promote further increases in *WUS* transcription. The positive feedback inherent in this circuit has

the consequence of turning on *WUS* robustly as sufficient signaling is achieved. Additional exploration of the model suggests this positive feedback biases *WUS* to be expressed in a bistable switch-like manner (see Computational Modeling in *SI Appendix*). Experimentally, *WUS* is expressed in a switch-like mode, expressed strongly in some cells while absent from directly adjacent cells. Switch behavior is important as it gives robust output even if input, such as cytokinin signaling, fluctuates.

In this study, relatively high levels of cytokinin were required to perturb the cytokinin signaling pathway and extend both the domain of cytokinin response and *WUS* expression. The requirement for high levels of cytokinin to disrupt the spatial distribution of cytokinin response is not surprising given that a multitude factors (Type-A ARRs, AHP6) act in a developmental context to suppress unrestrained signaling and maintain a stereotyped developmental pattern of response. Even at the relatively high levels of cytokinin used in this study, the spatial domain of cytokinin response changed only slightly when compared to its unperturbed pattern, as monitored by the *pTCS*::*GFP* reporter. The relatively sharp expression profile of the cytokinin receptors may also explain why strong cytokinin perturbations were required to extend the domain of cytokinin response and *WUS* expression. Cytokinin response within a given cell is the output of receptor concentration and cytokinin. Therefore cells with only low levels of receptor, such as cells farther away from the center of the SAM, require high levels of cytokinin to have significant cytokinin response.

This study shows that cytokinin response regulates *WUS* expression through both CLV-dependent and CLV-independent mechanisms. Given that *WUS* promotes cytokinin response (21), our experimental and computational modeling data suggests that *WUS* and cytokinin signaling interact through multiple positive-feedback loops which ultimately control stem cell number in the SAM. Future studies will show whether cytokinin acts as signaling cue to relay information between cells in different domains of the SAM as cells comprising different zones change during growth of the plant.

Materials and Methods

Plant Materials and Reporter Constructs. *clv1–11*, *clv3–2*, and *clv2–1* alleles in L-*er* background have been previously described (36, 37). The *pWOL*::*GFP* line in Columbia (Col-0) background has been previously described (38), and it recapitulates expression patterns observed in the shoot and root via in situ hybridization (30). The *pARR5*::*GFP* line in WS ecotype has been previously described (13). The *pWUS*::*GFP-ER* and *pCLV3*::*GFP-ER* lines have also been previously described (10, 34) (for details of other lines see *SI Materials and Methods*).

Plant Growth and Cytokinin Treatment Conditions. Plants were grown as previously described (29). Cytokinin treatments with N6-benzylaminopurine (BAP; Sigma–Aldrich Co.) were performed as described (23) except that shoots were sprayed with the respective solutions (for details see *SI Materials and Methods*).

Quantitative Real-Time PCR (qRT-PCR). Quantitative real-time PCR (qRT-PCR) was performed with Roche Universal Probe Library hydrolysis probes. Each sample represents tissue harvested from 50 two-week-old seedlings just transitioned to flowering (for details see *SI Materials and Methods*).

ACKNOWLEDGMENTS. We thank P. Benfey for the *pWOODENLEG*::*GFP* line referred to here as the *AHK4* reporter, *pAHK4*::*GFP*. The *pARR5*::*GFP* line was provided by J. Kieber and Y. Helariutta provided the *pAHP6*::*AHP6-GFP* line. Cytokinin receptor mutants were provided by T. Kakimoto. We thank the Computable Plant group (http://computableplant.org/) including H. Jönsson, M. Heisler, A. Roeder, and P. Tarr for comments on the manuscript; K. Sugimoto for advice with RT-PCR analysis of treated root explants; A. Martin for sample collection of cytokinin serial dilutions; and A. Garda for technical support. This study was supported by National Science Foundation grants IOS-0846192 and IOS-0544915 to E.M.M.

1. Sablowski R (2007) The dynamic plant stem cell niches. *Curr Opin Plant Biol* Dec;10:639–644.
2. Fletcher JC, Meyerowitz EM (2000) Cell signaling within the shoot meristem. *Curr Opin Plant Biol* 3:23–30.
3. Clark SE (2001) Cell signaling at the shoot meristem. *Nat Rev* Mol Cell Biol 2:276–284.
4. Haecker A, Laux T (2001) Cell-cell signaling in the shoot meristem. *Curr Opin Plant Biol* 4:441–446.
5. Clark SE, Williams RW, Meyerowitz EM (1997) The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. *Cell* 89:575–585.
6. Fletcher JC, Brand U, Running MP, Simon R, Meyerowitz EM (1999) Signaling of cell fate decisions by CLAVATA3 in *Arabidopsis* shoot meristems. *Science* 283:1911–1914.
7. Brand U, Fletcher JC, Hobe M, Meyerowitz EM, Simon R (2000) Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by CLV3 activity. *Science* 289:617–619.
8. Mayer KF, Schoof H, Haecker A, Lenhard M, Jurgens G, Laux T (1998) Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. *Cell* 95:805–815.
9. Schoof H, Lenhard M, Haecker A, Mayer KF, Jurgens G, Laux T (2000) The stem cell population of *Arabidopsis* shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* 100:635–644.
10. Reddy GV, Meyerowitz EM (2005) Stem-cell homeostasis and growth dynamics can be uncoupled in the *Arabidopsis* shoot apex. *Science* 310:663–667.
11. Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp Soc Exp Biol* 54:118–130.
12. Long JA, Moan EI, Medford JI, Barton MK (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of *Arabidopsis*. *Nature* 379:66–69.
13. Yanai O, et al. (2005) Arabidopsis KNOXI proteins activate cytokinin biosynthesis. *Curr Biol* 15:1566–1571.
14. Jasinski S, et al. (2005) KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. *Curr Biol* 15:1560–1565.
15. Tucker MR, Laux T (2007) Connecting the paths in plant stem cell regulation. *Trends Cell Biol* 17:403–410.
16. Kurakawa T, et al. (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* 445:652–655.
17. Kakimoto T (2003) Perception and signal transduction of cytokinins. *Annu Rev Plant Biol* 54:605–627.
18. Muller B, Sheen J (2007) Advances in cytokinin signaling. *Science* 318:68–69.
19. To JP, et al. (2004) Type-A *Arabidopsis* response regulators are partially redundant negative regulators of cytokinin signaling. *Plant Cell* 16:658–671.
20. To JP, Kieber JJ (2008) Cytokinin signaling: Two-components and more. *Trends Plants Sci* 13:85–92.
21. Leibfried A, et al. (2005) WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. *Nature* 438:1172–1175.
22. Laux T, Mayer KF, Berger J, Jurgens G (1996) The WUSCHEL gene is required for shoot and floral meristem integrity in *Arabidopsis*. *Development* 122:87–96.
23. Lindsay DL, K. Vipen V, Bonham-Smith PC (2006) Cytokinin-induced changes in CLAVATA1 and WUSCHEL expression temporally coincide with altered floral development in *Arabidopsis*. *Plant Science* 170:1111–1117.
24. Clark SE, Running MP, Meyerowitz EM (1993) CLAVATA1, a regulator of meristem and flower development in *Arabidopsis*. *Development* 119:397–418.
25. DeYoung BJ, Bickle KL, Schrage KJ, Muskett P, Patel K, Clark SE (2006) The CLAVATA1-related BAM1, BAM2 and BAM3 receptor kinase-like proteins are required for meristem function in *Arabidopsis*. *Plant J* 45:1–16.
26. Kayes JM, Clark SE (1998) CLAVATA2, a regulator of meristem and organ development in *Arabidopsis*. *Development* 125:3843–3851.
27. Muller R, Bleckmann A, Simon R (2008) The receptor kinase CORYNE of *Arabidopsis* transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1. *Plant Cell* 20:934–946.
28. Muller B, Sheen J (2008) Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature* 453:1094–1097.
29. Gordon SP, Heisler MG, Reddy GV, Ohno C, Das P, Meyerowitz EM (2007) Pattern formation during de novo assembly of the *Arabidopsis* shoot meristem. *Development* 134:3539–3548.
30. Mahonen AP, et al. (2006) Cytokinins regulate a bidirectional phosphorelay network in *Arabidopsis*. *Curr Biol* 16:1116–1122.
31. Gallois JL, Woodward C, Reddy GV, Sablowski R (2002) Combined SHOOT MERISTEMLESS and WUSCHEL trigger ectopic organogenesis in *Arabidopsis*. *Development* 129:3207–3217.
32. Gallois JL, Nora FR, Mizukami Y, Sablowski R (2004) WUSCHEL induces shoot stem cell activity and developmental plasticity in the root meristem. *Genes Dev* 18:375–380.
33. Kim HJ, et al. (2006) Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in *Arabidopsis*. *Proc Natl Acad Sci USA* 103:814–819.
34. Jonsson H, et al. (2005) Modeling the organization of the WUSCHEL expression domain in the shoot apical meristem. *Bioinformatics* 21:i232–240.
35. Geier F, Lohmann JU, Gerstung M, Maier AT, Timmer J, Fleck C (2008) A quantitative and dynamic model for plant stem cell regulation. *PLoS ONE* 3:e3553.
36. Dievart A, et al. (2003) CLAVATA1 dominant-negative alleles reveal functional overlap between multiple receptor kinases that regulate meristem and organ development. *Plant Cell* 15:1198–1211.
37. Clark SE, Running MP, Meyerowitz EM (1995) CLAVATA3 is a specific regulator of shoot and floral meristem development affecting the same processes as CLAVATA1. *Development* 121:2057–2067.
38. Birnbaum K, et al. (2003) A gene expression map of the *Arabidopsis* root. *Science* 302:1956–1960.